Development of a New Toxicity Test Method Using a Bio-dipstick

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ABSTRACT

The toxicity is an important property of environmentally considerate lubricants. To determine this property, numerous toxicity test methods have been developed using sophistical devices. Some of these tests have adapted to evaluating the toxicity properties of lubricants, while others have required some modification or do not work in lubrication applications. Currently, the OECD 203 guideline 203, fish, acute toxicity test, is widely used to evaluate the toxicity of lubricants using trout. This toxicity test method also requires special biological knowledge and a well- equipped laboratory. For this reason, most toxicity test methods cannot be performed in the petroleum laboratory.

The bio-dipstick is a formation of agar medium and was designed to monitoring of microbial contamination in various industrial environments. Recently, this bio-dipstick has been also successfully used for screening the initial inoculums conditions for the aquatic biodegradation tests. In a similar approach, a potential toxicity test method for lubricants was studied using a bio-dipstick. The test results showed that the proposed test method was able to differentiate the toxicity level of lubricants and provided an acceptable test precision. In addition, it gave a good correlation with the results of OECD guideline 203 test. It appears that this toxicity test method can screen the toxicity of lubricants within very short period.

This paper will be presented how to develop the toxicity test method and its test results.

INTRODUCTION

Environmental safety and compliance has recently become the most significant worldwide issue. In the recent years there has been an increasing interest in Environmentally Considerate Lubricant (ECL) products which are formulated with low toxic and more biodegradable materials than conventional petroleum-based lubricants. Especially, the toxicity of lubricant is an essential property of ECL products with their biodegradability. Generally, the toxicity is defined as the degree to which a chemical substance can damage an organism such as an animal including human, bacterium, or plant, and can be measured by its effects on the organism. Today, there are many toxicity test methods available for evaluating the degree of toxicity of lubricants. Among

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them, the Organization for Economic Cooperation and Development (OECD) aquatic toxicity test methods have been widely used for the lubrication applications. These toxicity test methods were originally designed to determine the effects of a substance on specified test species (i.e., fish, micro-organism, etc.) under laboratory test conditions. Most aquatic toxicity test methods express their test results by LC 50 which is the lethal concentration of the chemical in air or water that kills 50 % of the test species in a given time. Few toxicity tests report the test results as an EC 50, which is a concentration estimated to cause an effort on a test end point in 50% of exposed population over a defined exposure period, to classify the toxicity level.

OECD 203 test method was designed to determine the degree of acute aquatic toxicity of chemicals by the mortalities of fishes[1]. The toxicity test is actually performed using the selected fishes (i.e., rainbow trout, etc.) which are exposed to the test chemicals for a period of 96 hours in the aquatic tank and determined concentration (LC50) which kill 50 % of the fish. In this test, the chemicals with low solubility water (i.e., petroleum-based oils, etc.) may not be possible to quantitatively determine the LC50. To resolve these types of problems, some methodologies use the water-accommodated fraction, part of oil that disperse or dissolves in water, although this is not a true reflection of the entire oil toxicity in water. This technique may also kill the fishes due to the blocking of oxygen supply and the surface tension of water. Currently, this test method is widely used to determine the toxicity of lubricants including bio-based hydraulic fluids. In this test, the LC 50 is greater than 1000 mg/L, it can be considered as a low toxicity which is a chemical property of the environmentally considerate lubricants.

OECD 209 test method is to determine the effects of a substance on micro-organisms from activated sludge by measuring their respiration rate under defined conditions in the presence of different concentrations of the test substance[2]. In a preliminary test, the toxic concentration range of the test substance is first established. Then, the test solutions incubated for 30 minutes (up to 3 hours) at 20 °C. The test results are expressed by the EC50 which is a 50 % effect concentration related to the oxygen consumption. This test method has been used to evaluate the toxicity of water-soluble, poorly soluble and volatile substances (i.e., lubricants, etc.) and to give early warning of possible harmful effects of chemicals on the aerobic treatment of wastewaters as well as indicating non-inhibitory concentrations of test substances to be used in the various tests for biodegradability.

Currently, the ASTM D-2 Subcommittee 12 on Environment is developing a new toxicity test method using a Microtox instrument[3]. This test method covers a procedure for the rapid evaluation of the toxicity of water accommodating extracts taken from lubricating fluids when exposed to freshwater or marine matrices on luminescent marine bacterium, Vibrio fisheri. In this test, the intensity of light is measured when the luminescent bacteria are exposed the toxic sample. Healthy bacteria emit a great amount of light, while, the more toxic sample has less light which produced by bacteria. The test results are expressed in EC50 which represents the calculated concentration of sample that would produce a 50 % reduction in light output (intensity) of exposed bacteria over specified time. High value of effective concentration indicates low

toxicity, while low values indicate high toxicity. Currently, this test method is widely used in the wastewater treatment center and chemical industries to monitor water quality and soil testing. But the test method is not yet used to evaluate the toxicity of lubricants due to the poor solubility of water. To resolve this problem, the ASTM D-2 Subcommittee 12 is studying a water-accommodated fraction technique to adopt the microtoxic procedure for lubricant applications.

All aquatic toxicity test methods used in lubricant applications require the good water solubility of lubricants to determine the toxicity level. Unfortunately, most petroleum based lubricants have very poor water solubility, and the test apparatus is normally not available in petroleum testing laboratories. For this reason, the toxicity of lubricants cannot be effectively determined in most case. Therefore, a new toxicity test method is needed to evaluate the toxicity level of lubricants within a short period. Especially, the lubricants must be evaluated their toxicity level to certify as an environmentally considerate lubricant.

TEST PROCEDURE DEVELOPED

A bio-dipstick is commercially made to detect the microbial growth in industrial environments such as cutting fluids, fuel tanks, and paint manufacturing[4]. U.S. Army has also used this bio-dipstick to monitor the inoculums for the biodegradation test and to evaluate the effective of bio-cide in diesel and JP-8 fuel[5,6]. This bio-dipstick has agar medium on both sides. The one slide is covered with triphenytetrazolium chloride (TTC) agar supporting the growth of most common bacteria. The other side is covered with Rose-Bengal agar which supports the growth of fungi. Figure1 shows a bio-dipstick and the model density charts for bacteria counting is posted in Figure 4. Using this device, the bacteria growth can be detected by a simple procedure. First, a bio-dipstick dips into a fluid tank or wetted by spraying of fluid. Then, place in a plastic container (tube). The test sample is kept in an incubator at 27-30 °C for 48 hrs. After incubation, the bio-dipstick was removed from its tube. Compare the density of the colonies growing on the medium with the model density charts[7] without actually counting colonies. The higher colonies numbers is more bacteria growth than lower colonies numbers. If the fluid is toxic, the bacteria are less growth on a bio-dipstick.

To develop a toxicity test for lubricants,   the test procedure was modified to measure the degree of toxicity. First, the test lubricant was coated or filmed on the bio-dipstick by spraying or a spatula. Then, the inoculums collected from the wastewater treatment center, was treated on the bio-dipstick coated with the test sample. This bio-dipstick was stored in an incubator at 30 °C for 72 hrs. After incubation, the bacteria were counted using the model density charts[7]. In this test, the degree of toxicity was determined based on the bacteria count in number of colony forming units (CFU)/ ml comparing with the model density chart and the proposed toxicity classification system which is shown in Table1. If the lubricant has low bacteria growth on the bio-dipstick, it classifies as a high toxic lubricant.

Table 1. Proposed Toxicity Classification

Toxicity Classification	Toxicity Level	Bacteria Growth Count, CFU/ml
1	Most toxic	$< 10^3$
2	Slightly toxic	10^3 - 10^4
3	Practically non-toxic	$\geq 10^5$

TEST SAMPLES

The lubricants are currently formulated with various types of oils and additives or thickening systems to meet specific applications. Their toxicity levels are also various with their chemical compositions and additive packages. For the study, twenty (20) samples were selected including a canola based cooking oil and a Clorox used in the household cleaning agent. They consist of eleven lubricating oils used in the ASTM D7373 round robin test, four military bio-based hydraulic fluids qualified under MIL-PRF-32073 specification[8], and three military lubricating greases (i.e., MIL-PRF-10924G[9], Mil-PRF-10924H[10], Mil-PRF-81322G[11]). The ASTM D 7373 round robin samples[12] have been used to conduct a study for evaluating the biodegradability and determining the test precision. Canola based cooking oil is known as a practically non-toxic oil, while Clorox is a toxic chemical which kills bacteria. Five bio-based hydraulic fluids including a round robin sample, qualified under MIL-PRF-32073 have been evaluated using OECD 203 test method, were selected to make a correlation with the new test method. Three grease samples were also included in this study to determine the applicability of this toxicity test. The inoculums also collected from local waste water treatment center were tested as a baseline study. These samples were fully formulated by the lubricant companies with different types of base oils, viscosity grades, and chemical additives. All of these lubricants are currently being used in the various mechanical systems. Table 2 identifies the test samples by codes and describes their base oil types, chemical materials, biodegradability and applications.

Table 2. Test Sample for Toxicity Test

Sample Code	Sample name	Description	Biodegradability, %, 28 days	Application
A	ASTM RR 7373-S-1	Bio-based oil	67.6	Tractor hydraulic fluid
B	7373-S-2	Mineral oil	35.1	Hydraulic fluid
C	7373-S-3	PAO 2 cSt	67.3	Base oil
D	7373-S-4	Mixed Bio-based oils	77.9	MIL-PRF-32073 Grade 3, Bio-based Hydraulic Fluid
E	7373-S-5	Water soluble	16.3	-

		polypropyleneglycol (PPG)		
F	7373-S-6	Mineral oil	34.1	Gear oil
G	7373-S-7	Mineral oil	35.0	Tractor hydraulic oil
H	7373-S-8	Bio-based oil	77.6	Hydraulic oil
I	7373-S-9	Mixed bio-based oil	52.0	Hydraulic oil
J	7373-S-10	PAO 4 cSt	53.4	Base oil
K	7373-S-11	Water insoluble , hydrocarbon soluble Polybutyleneglycol (PBG)	57.9	-
L	Canola oil	Canola oil	83.0	Cooking oil
M	Clorox	Sodiumhypochlorite &Sodium hydroxide	ND	Laundry
N	MIL-PRF-32073 Grade 1	Synthetic ester	74	Bio-based hydraulic fluid
O	MIL-PRF-32073 Grade 2	Synthetic ester	62	Bio-based hydraulic fluid
P	MIL-PRF-32073 Grade 4	Vegetable-based oil	73	Bio-based hydraulic fluid
Q	MIL-PRF-32073 Grade 5	Vegetable- based oil	68	Bio-based hydraulic fluid
R	MIL-PRF-10924G	Grease, PAO with Lithium complex	30	Automotive
S	MIL-PRF-81322	Grease, PAO with Clay	30	Aviation
T	MIL-PRF-10924H	Grease, Polyol ester and PAO with Lithium complex	61	Automotive
U	Inoculums	Activated Sludge	ND	Wastewater Treatment Center

TEST RESULTS AND DISCUSSIONS

A summary of the test results is presented in Table 3. The test samples were identified by designated codes. Data were generated based on the bacteria growth counting on the bio-dipstick using the model density chart, and converted to the toxicity levels based on the proposed toxicity classification. To check the test precision, the triplicate tests were conducted using different batch of inoculums collected at the different day.   The test results showed that the bio-dipstick test can differentiate the toxicity levels of lubricants and provides good test precision. The test condition (i.e., 30 C, 72 hours) associated with this toxicity test was adequate to growth the bacteria on the tested samples. In this test, the bacteria were counted ranging from zero to $1x10^5$ colony forming units (CFU)/ml in the tested lubricants. The inoculums (sample U) showed the bacteria growth of

$1x10^5$ CFU/ml on the bio-dipstick. In the other samples, the bio-dipstick coated with cooking oil (sample L) also showed the bacteria growth of $1x10^5$ CFU/ml, but the bio-dipstick coated with Clorox (sample M) did not growth any bacteria. In fact, the cooking oil is known as nontoxic oil, while the Clorox is toxic chemical. Their test results are shown in Figure 2 which clearly demonstrated that the test procedure can differentiate the non-toxic and toxic materials.

Lubricating oils were formulated with base oils and additives to meet the requirements of various mechanical systems. The toxicity levels of these lubricating oils depend on the source of base oils and their additive packages. Generally, some additives can significantly increase to the toxicity of lubricating oils. Table 3 showed that all bio-based oils were rated by Class 3 which is denoted as the practically non-toxic chemicals, while the mineral oils were rated by Class 2 which is the slightly toxic chemicals. The petroleum based synthetic oils including Polyalpaolefine (PAO) oils were also rated as Class 2. The test results clearly indicated that the lubricating oils having high biodegradability (greater than 60%) tend to have a very low toxicity.

Three different types of lubricating greases were tested to determine their suitability in this test procedure. One is a biodegradable grease formulated with Polyol ester, while the other greases were PAO based greases. For the test, a very thin film was applied on both sides of growth medium because the test results cannot show well on the test medium when applied thicker grease film on the medium. Table 3 show that the biodegradable grease (sample T) has a low toxicity level (Class 3), while two other PAO based greases (sample R and S) were rated as Class 2. These test results were very similar to the results obtained from lubricating oils. It appeared that the lubricating grease can be also tested in this procedure if very thin grease film is applied on the test specimen.

The test precision is a very important factor for the development of a new test procedure. Generally, the microbiology test such as biodegradation test has very poor precision due to the various of inoculums sources and unknown microorganisms' behavior. In similar approach, for the test, the inoculums were collected at three different times and each toxicity test was conducted at different day to check their precision. Table 3 show that the test precision was excellent and almost identical data were produced for each sample. In addition, it has generated meaningful data which agree to the known toxicity data. Due to the nature of the test results and limited data, the test precision cannot be determined by the ASTM D6300[13]. In an effort, the repeatability of test procedure was calculated as 91% based on the duplicate results.

Table 3. The Toxicity Test Results using a Bio-dipstick

Sample Code	Description	Bacteria growth count, 72 hours			Proposed Toxicity Classification
		1	2	3	
A	Bio-based oil	10^5	10^5	10^5	3
B	Mineral oil	10^4	10^4	10^4	2

C	PAO 2 cSt	10^5	10^4	10^4	2
D	Bio-based oil	10^5	10^5	10^5	3
E	Water insoluble , hydrocarbon soluble Polybutyleneglycol	10^6	10^5	10^5	3
F	Mineral oil	10^4	10^4	10^4	2
G	Mineral oil	10^4	10^4	10^5	2
H	Bio-based oil	10^5	10^4	10^5	3
I	Mixed bio-based oil	10^5	10^5	10^5	3
J	PAO 4 cSt	10^4	10^4	10^4	2
K	Water insoluble polypropyleneglycol	10^5	10^5	10^5	3
L	Canola cooking oil	10^5	10^5	10^5	3
M	Sodiumhypochlorite &Sodium hydroxide	0	0	0	1
N	Synthetic ester-based oil	10^5	10^5	10^5	3
O	Synthetic ester-based oil	10^5	10^5	10^5	3
P	Bio-based oil	10^5	10^5	10^5	3
Q	Bio-based oil	10^5	10^5	10^5	3
R	Grease, PAO with Lithium complex	10^3	10^3	10^3	2
S	Grease, PAO with Clay	10^3	10^3	10^3	2
T	Grease, Polyol ester and PAO with Lithium complex	10^5	10^5	10^5	3
U	inoculums	10^5	10^5	10^5	3

A correlation study was also conducted using military bio-based hydraulic fluids qualified under MIL-PRF-32073, Hydraulic fluid, bio-based. These fluids have been tested according to the OECD 203 test and met the specification requirement (LC50: minimum 1000mg/L) which is equivalent to the ASTM hydraulic fluid ecotoxicity classification (Tw1)[14] that is the lowest toxicity level. Five military bio-based fluids (D, N, O, P, Q,) were also evaluated according to the new test toxicity procedure. All of these fluids were rated as Class 3 which is equivalent to Tw1. These test results are shown in Table 4. It clearly indicated that the low toxicity level of OECD 203 test (above 1000 mg/L) correlates to the level 3 of toxicity classification obtained from bio-dipstick test. Therefore, the bio-dipstick test can be used as a screen test for OECD 203 test.

Table 4. Correlation between OECD 203 test and Bio-dipstick Test

Sample Code	OECD 203 Test Result, LC50, mg/L*	ASTM Acute Ecotoxicity Classification	Bio-Dip Stick Test result (Toxicity classification Number)
N	3272	Tw1	3
O	2148	Tw1	3
D	2160	Tw1	3
P	10,000	Tw1	3
Q	8505	Tw1	3

*Minimum LC50 of MIL-PRF-32073 specification requirement is 1000 mg/L

CONCLUSIONS

On the basis of the study completed to date, a new toxicity test method was developed using a bio-dipstick technique. The test procedure provides a meaningful toxicity data within a very short period and the repeatability of the test method was found as 91% which is acceptable. Most bio-based oils and grease were rated as Class 3 which is the practically non-toxic chemicals. Petroleum based oil and derived synthetic oil such as PAO were rated as Class 2. The toxicity level of cooking oil (canola oil) was found as Class 3, while Clorox was rated as Class 1 which is the toxic chemical. Based on the test results, the test method can differentiate the toxicity level of lubricants.

A correlation was found between OECD 203 test method and bio-dipstick procedure using military bio-based hydraulic fluids qualified under MIL-PRF-32073 specification. It was found that more than 1000 mg/L of LC 50 from OECD 203 is equivalent to Class 3 obtained from the bio-dipstick. This toxicity rating also agreed with the ASTM non-toxic classification (Tw1) which is required for environmental considerate lubricants. Therefore, the bio-dipstick test can be used as a screen toxicity test for OECD 203 test.

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Figure 1. New Bio-dipstick

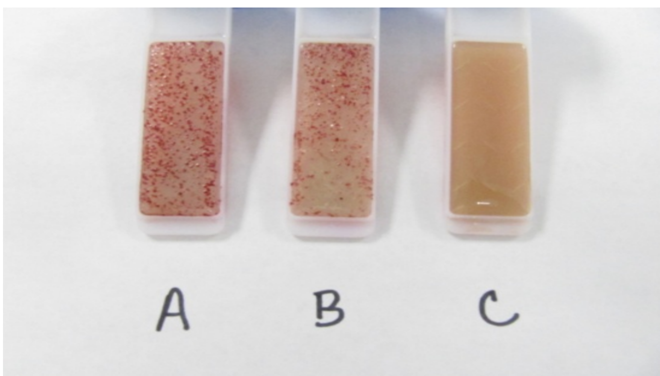

Figure. 2 Toxicity test results from Sample L, M, and U

A: Activated Sludge B. Canola based cooking oil C. Clorox (Laundry)

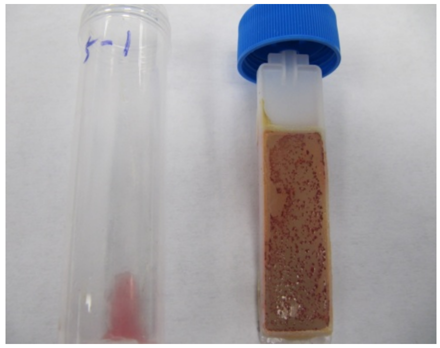

Figure 3. Toxicity test result for Biodegradable grease T

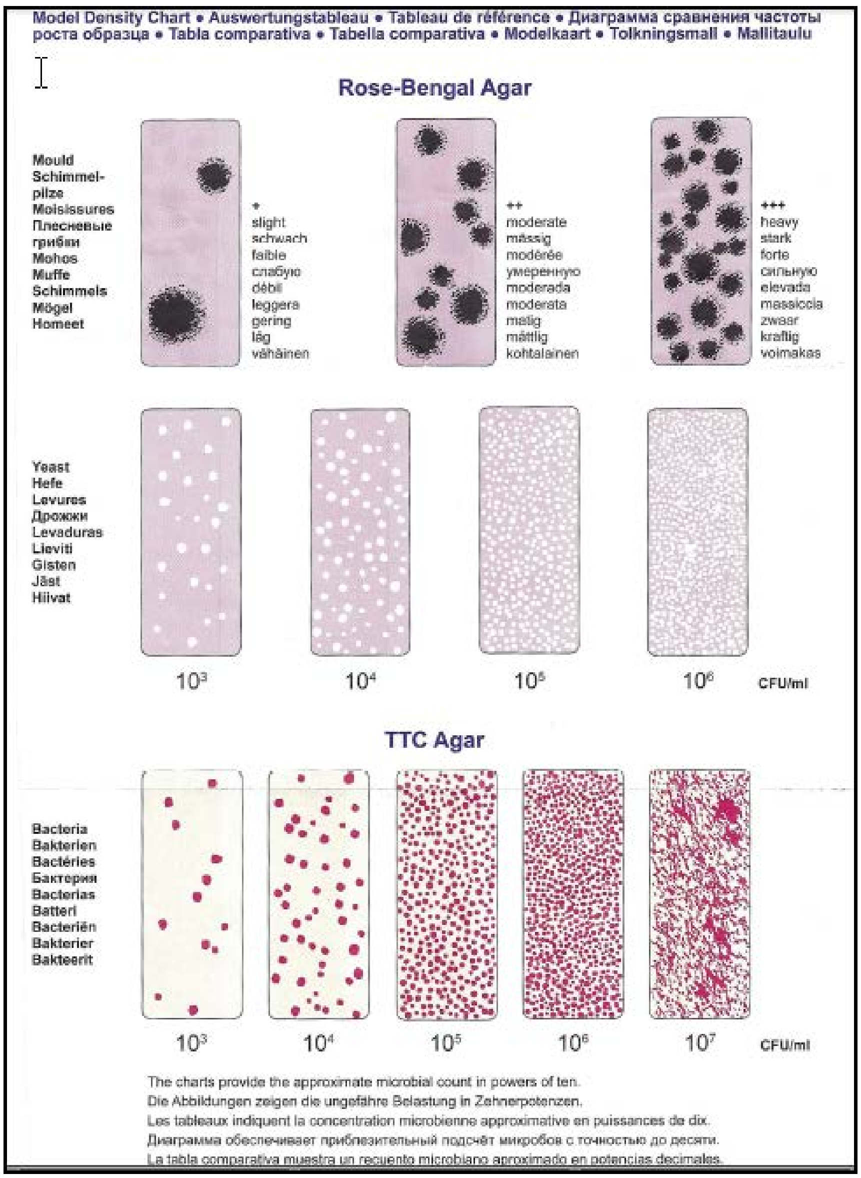

Figure 4. Model Density Chart